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Characterization of rat brain NCAM mRNA using DNA oligonucleotide probes

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A number of different isoforms of the neural cell adhesion molecule (NCAM) have been identified. The difference between these is due to alternative splicing of a single NCAM gene. In rat brain NCAM mRNAs with sizes of 7.4, 6.7, 5.2, 4.3 and 2.9 kb have been reported. We have synthesized six DNA oligonucleotides, that hybridize to different exons in the NCAM gene. Furthermore we have constructed three oligonucleotides, that exclusively hybridize to mRNAs lacking certain exons, by letting them consist of sequences adjacent to both sides of the splice sites. By means of these probes we have characterized the five NCAM mRNAs in rat brain.

1. INTRODUCTION

NCAM is a family of closely related cell surface glycoproteins involved in cell-cell adhesion. In rodent brain NCAM is present as three major glycosylated polypeptides with sizes of 190, 135 and 120 kDa, in the following referred to as NCAM-A, NCAM-B and NCAM-C (for review see [1]). The diversity between the NCAM polypeptides is mainly due to differences in the C-terminal parts. NCAM-A and NCAM-B are both integral membrane proteins; the difference in size is due to the presence of an extra domain in the cytoplasmic region of NCAM-A. NCAM-C lacks a membrane spanning domain and is attached to the membrane via a phosphatidylinositol anchor [2]. Although originally described as a neural protein, NCAM is also expressed by other cell types such as skeletal and smooth muscle cells (for review see [3]).

There is only one gene for NCAM in the haploid genome [4] and the different NCAM isoforms are generated by differential splicing and alternative polyadenylation [5]. There are five different NCAM mRNA size classes of 7.4, 6.7, 5.2, 4.3 and 2.9 kb in adult rat brain [6]. More than 20 different exons are described that code for the distinct NCAM polypeptides [5,7–11]. In the genome several small exons exist that are spliced between exon 12 and exon 13. In the following, when referring to exon numbers the nomenclature of [12] for chicken NCAM is used. These include a 15 bp exon called exon a, found in both mouse brain and muscle [11], often followed by an unusually short exon of only three nucleotides, AAG. Another exon, SEC, that presumably generates a secreted form of NCAM and is demonstrated in human muscle and mouse brain [13] may also be inserted between exon 12 and 13. An exon, called Pi, consisting of 30 bp, can be inserted between exon 7 and 8 [8] (see Fig. 1). These findings indicate that the NCAM mRNA pattern is much more complex than first assumed, and that several distinct mRNA forms may be hidden in the different size classes. To further clarify the NCAM mRNA pattern in rat brain, we have constructed oligonucleotides, that are specific for various NCAM exons. We show that it is possible to make probes, that specifically recognize mRNAs, that lack exons at certain splice sites, by making probes consisting of sequences that hybridize both to the left and right side of the splice site (see Fig. 1). Furthermore, we demonstrate that the 4.3 kb mRNA size class consists of at least two distinct mRNA forms, one form containing sequences from exon 18 and another form lacking exon 18, but containing adjacent parts of exon 17 and 19. We also demonstrate that exon a is expressed in 6.7, 5.2 and 2.9 kb mRNAs, but not in the 7.4 kb mRNA. Finally, we confirm the findings of [8] that all NCAM mRNA size classes consist of two forms: one containing the Pi-exon and another lacking this exon.

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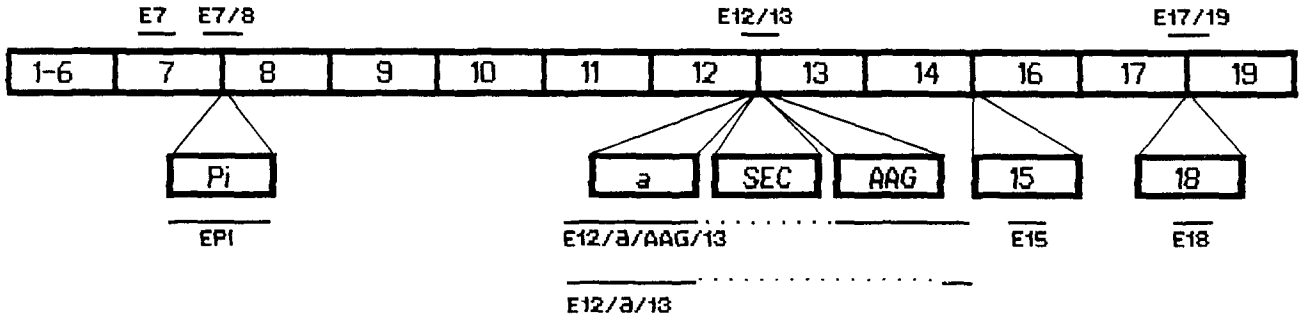

Fig. 1. NCAM exons in rodent brain. The localization of the synthetic oligonucleotides described in the text is indicated by bars.

2. MATERIALS AND METHODS

2.1. *Oligonucleotides*

Oligonucleotides (30–48 nucleotides) specific for different NCAM mRNA were constructed using published sequences of NCAM cDNA clones (see Fig. 1). An oligonucleotide specific for exon 18 in mouse (E18 probe) was constructed using the sequence published in [14] (covering nucleotides 567–597). An oligonucleotide specific for exon 15 was constructed from [15] (E15 probe, covering nucleotides 2262–2294). From the rat NCAM cDNA sequence published in [6], an oligonucleotide hybridizing to exon 7, which is contained in all NCAM mRNA classes (E7 probe, covering nucleotides 1177–1224), was constructed. From the same sequence an oligonucleotide specific for exon 17 followed by exon 19 (E17/19 probe, covering nucleotides 2648–2677) was constructed. Oligonucleotides specific for NCAM mRNA that either possess or lack the Pi exon were also constructed (EPi probe, nucleotides 1271–1300 and E7/8 probe, nucleotides 1257–1270 + 1301–1315) using the sequence given in [6]. Oligonucleotides were constructed from the sequence described in [11] specific for exon a + AAG (E12/a/AAG/13 probe, nucleotides 1946–1959 + exon a + AAG + 1960–1969), exon a without AAG (E12/a/13 probe, nucleotides 1946–1959 + a + 1960–1972), and mRNA lacking exon a and AAG (E12/13 probe, nucleotides 1946–1976).

Oligonucleotides were synthesized on a Biosearch 8750 DNA-synthesizer and labelled with [^{32}P]dATP (NEN) using a DNA tailing kit from Boehringer Mannheim.

2.2. *Northern blotting analysis*

Total RNA was extracted from rat brain and rat liver by a phenol-chloroform method [16,17] and then passed through an oligo(dT)-cellulose column (Pharmacia) according to [18].

Poly(A$^+$) RNA was denatured in 50% formamide, 6% formaldehyde in H_2O by heating at 56°C for 15 min and fractionated by electrophoresis through 0.8% agarose gel containing 6.15% formaldehyde. An RNA standard (9.5, 7.5, 4.4, 2.4, 1.4 and 0.24 kb, BRL) was applied on the gel. Fractionated RNA was blotted onto nitrocellulose filters (Schleicher & Schüll) by capillary blotting overnight in 20 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate). Prehybridization (2 h) and hybridization (18–20 h) were performed at 60–65°C in 4 × SSC, 0.1% sodium dodecyl sulphate (SDS), 0.1% Denhardt's solution (2% Denhardt's solution = 2% Ficoll, 2% bovine serum albumin, 2% polyvinylpyrrolidone), 0.2 mM EDTA, 200 μg poly A/ml, 0.06% tetrasodium diphosphate. After hybridization filters were washed in 1 × SSC, 0.1% SDS at hybridization temperature.

3. RESULTS

The specificity of the different oligonucleotides was tested by Northern blotting using poly(A$^+$) RNA isolated from rat brain at different stages of development and from adult rat liver. The results obtained using the probes are shown in Figs 2 and 3.

The E7 probe recognized all five NCAM mRNA classes (7.4, 6.7, 5.2, 4.3, and 2.9 kb) in rat brain (see Fig. 2, lanes 1 and 2). It did not recognize any mRNAs in adult rat liver which was run concomitantly in all experiments. When hybridizing the E7 probe to brain mRNA isolated from different stages of development, the same developmental changes in NCAM mRNAs as described in [6,19] were seen (data not shown). The 4.3 kb mRNA class was only present in small amounts and usually observed in rat brain postnatal days 4 and 10, where the total NCAM mRNA amount was highest. The 4.3 kb mRNA usually became more apparent after longer exposure (2–3 days).

The E18 probe hybridized to the 7.4 kb mRNA class as expected. Furthermore the E18 probe also hybridized to the 4.3 kb mRNA class (see Fig. 2, lane 3), in-

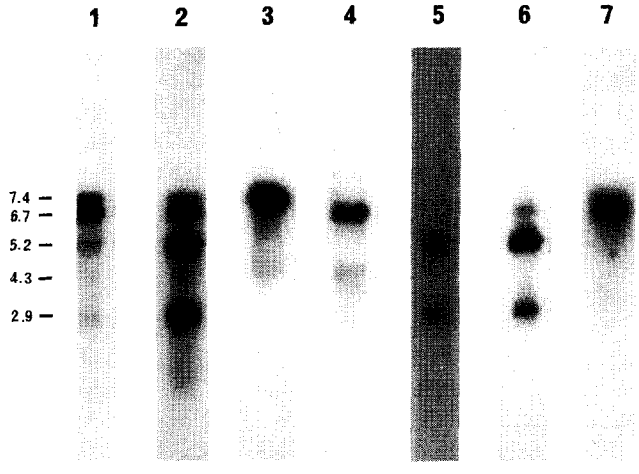

Fig. 2. Northern blotting analysis of NCAM mRNA. 10 μg of poly(A$^+$) RNA were loaded per lane. E7 probe hybridized to mRNA from postnatal day 10 rat brain is shown in lane 1, and to mRNA from adult rat brain in lane 2, E18 probe hybridized to postnatal day 4 rat brain mRNA (lane 3), E17/19 probe hybridized to postnatal day 4 rat brain mRNA (lane 4), E15 probe hybridized to adult rat brain mRNA (lane 5), E12/a/AAG/13 probe hybridized to postnatal day 10 rat brain mRNA (lane 6), E12/13 probe hybridized to postnatal day 10 rat brain mRNA (lane 7). The sizes of the hybridizing species are indicated in kb to the left.

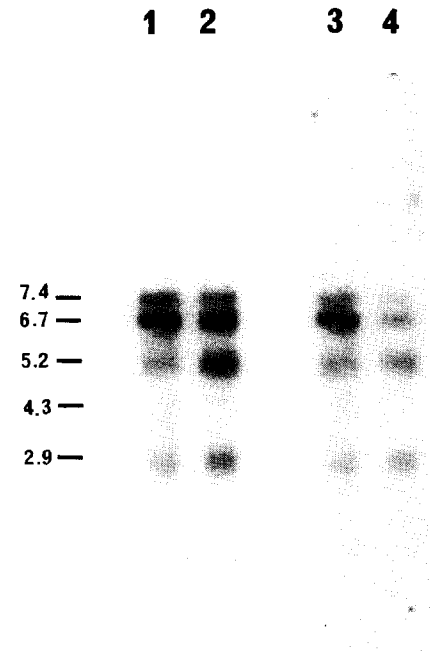

Fig. 3. Northern blotting analysis of NCAM mRNA using EPi and E7/8 oligonucleotide probes. Lanes 1 and 3, postnatal day 10 rat brain RNA; 2 and 4, adult rat brain RNA. Lanes 1 and 2, EPi probe, 2 and 4, E7/8 probe. The sizes of the hybridizing species are indicated in kb to the left.

dicating that exon 18 sequences also are contained in the 4.3 kb mRNA.

The E17/19 probe hybridized to the 6.7 and 4.3 kb mRNA classes (see Fig. 2, lane 4), whereas no hybridization to the 7.4 kb mRNA could be seen. This shows that even though the 7.4 kb mRNA contains exon 17 and 19 to which the E17/19 probe hybridizes, the E17/19 probe is specific for mRNAs lacking exon 18 at the chosen conditions.

The E15 probe hybridized to the 5.2 and 2.9 kb mRNA classes (Fig. 2, lane 5).

The E12/a/AAG/13 probe hybridized to 6.7, 5.2 and 2.9 kb mRNAs. No hybridization to the 7.4 kb mRNA could be seen (see Fig. 2, lane 6). The hybridization to 5.2 and 2.9 kb mRNAs was relatively more intense than the hybridization to the 6.7 kb mRNA (compare lane 1 with lane 6). This indicates that a relatively major fraction of the 5.2 and 2.9 kb mRNAs contains exon a/AAG, whereas the exon a/AAG-containing fraction in the 6.7 kb mRNA class is only a minor fraction.

The E12/a/13 probe did not hybridize to anything (data not shown), indicating that exon a usually is followed by the triplet exon, AAG.

With *the E12/13 probe* hybridization to the 7.4 and 6.7 kb mRNAs was seen (see Fig. 2, lane 7). In some experiments faint bands of 5.2 and 2.9 kb could also be observed.

The EPi probe hybridized to all five mRNA classes from postnatal day 10 and adult rat brain, even though the 4.3 kb band was very faint. No hybridization could be observed with the EPi probe in embryonal day 18 and postnatal day 4 rat brain, indicating that expression of NCAM mRNAs containing the Pi exon increases during development (see Fig. 3, lanes 1 and 2). These findings correlate with the findings of [8].

The E7/8 probe hybridized to the same mRNA classes as the EPi probe, except the 4.3 kb mRNA. The developmental pattern was, as expected, reversed; thus, the expression of NCAM mRNAs lacking the Pi-exon decreased during development (see Fig. 3, lanes 3 and 4).

No hybridization could be seen to the 4.3 kb mRNA class with the E7/8, the E12/a/AAG/13 probe or the E12/13 probe. This may be due to the very low amount of this mRNA population. The results obtained using the different probes are summarized in Table I.

4. DISCUSSION

Our results show that *the 7.4 kb mRNAs* contain exon 18. Some 7.4 kb mRNAs contain exon Pi and some do not. No 7.4 kb mRNA contains exon a/AAG or exon 15.

The 6.7 kb mRNA class contains exon 17 and 19, but not exon 18 as shown with the E17/19 probe. Neither does it contain exon 15. The 6.7 kb mRNA class seems

Table I

The result of hybridization with the indicated probes to the various NCAM mRNA species

mRNA class (kb)	Probes								
	E7	E7/8	EPi	E12/13	E12/a/AAG/13	E12/a/13	E15	E17/19	E18
7.4	+	+	+	+	–	–	–	–	+
6.7	+	+	+	+	+	–	–	+	–
5.2	+	+	+	(+)	+	–	+	–	–
4.3	(+)	?	(+)	?	?	–	–	(+)	(+)
2.9	+	+	+	(+)	+	–	+	–	–

+ indicates reaction in all experiments performed; (+) indicates a weak reaction not always observed in individual experiments

to consist of different forms, some containing exon Pi and/or exon a/AAG and some not. If the insertion of exon Pi and exon a/AAG are independent events at least four different isoforms of 6.7 kb mRNA can be generated: one form containing both exons, one containing only exon Pi, one containing only exon a/AAG, and finally one containing neither exon Pi nor exon a.

The 5.2 kb mRNA class contains exon 15, but not exons 17, 18 or 19. This mRNA class may also consist of different combinations of exon Pi and exon a/AAG since the E7/8, EPi, E12/13 and E12/a/AAG/13 probes all hybridize to this mRNA class. Gower et al. [13], who identified exon SEC as an exon inserted between exon 12 and 13 showed that this exon is associated with the 5.2 kb mRNA in mouse brain. Since both the E12/13 and E12/a/AAG/13 probes exclude the presence of exon SEC, only part of the 5.2 kb mRNA seems to contain exon SEC.

The 4.3 kb mRNA class showed hybridization to both the E18 and E17/19 probes. Since these two probes are constructed to be mutually exclusive, this indicates that the 4.3 kb mRNA class consists of at least two different forms, one possessing and one lacking exon 18 sequences. Exon 18 is 801 bp long in mice [15]. The difference in size between an mRNA that lacks exon 18 and one that contains the total exon 18 is too large for the two forms to colocalize in the same band on a Northern blot if they are otherwise identical. Therefore it is suggested that the two 4.3 kb mRNA forms consist of one that totally lacks exon 18 and one that possesses part of it. The EPi probe also hybridized to the 4.3 kb mRNA class.

The 2.9 kb mRNA contains the same exons as shown for the 5.2 mRNA class indicating that also this class consists of multiple forms.

The 7.4 and 6.7 kb mRNAs encode the NCAM-A and NCAM-B polypeptides, respectively. The 5.2 kb presumably encodes the secreted NCAM form and NCAM-C. The 2.9 kb mRNA encodes the NCAM-C polypeptide. No correlation between the 4.3 kb mRNA and any NCAM polypeptide has yet been found.

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